A simple dried blood spot method for therapeutic drug monitoring of the tricyclic antidepressants amitriptyline, nortriptyline, imipramine, clomipramine, and their active metabolites using LC-MS/MS

E.J.J. Berm [a,*], J. Paardekooper [b], E. Brummel-Mulder [b], E. Hak [c], B. Wilffert [a,d], J.G. Maring [b,**]

[a] Dept. of Pharmacy, section Pharmacotherapy and Pharmaceutical Care, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands
[b] Laboratory for Drug Analysis &Toxicology, Diaconessen Hospital Meppel& Bethesda Hospital, Hoogeveenseweg 38, 7943 KA, Meppel, the Netherlands
[c] Dept. of Pharmacy, section Pharmaco Epidemiology and Pharmaco Economics, University of Groningen, Antonius Deusinglaan 1, 9713 AV Groningen, the Netherlands
[d] Dept. of Clinical Pharmacy and Pharmacology, University Medical Center Groningen, P.O. Box 30.001, 9700RB, Groningen, the Netherlands

ARTICLE INFO

ABSTRACT

Therapeutic drug monitoring (TDM) of tricyclic antidepressants (TCAs) is considered useful in patients with major depressive disorder, since these drugs display large individual differences in clearance, and the therapeutic windows of these drugs are relatively small. We developed an assay for determination of amitriptyline (ATP), nortriptyline (NTP), imipramine (IMP), desipramine (DSP) clomipramine (CMP) and desmethyl-clomipramine (DCMP) in dried blood spots (DBS). A fast and robust LC-MS/MS method was developed and analytically validated for simultaneous determination of ATP, NTP, IMP, DSP, CMP, and DCMP in DBS. Six mm circles were punched out from DBS collected on Whatman DMPK-C paper and mixed with acetonitrile: methanol 1:3 containing the internal standard. The extract was analyzed by LC-MS/MS. Total LC-MS/MS runtime was 4.8 min. The assay was linear in the range 20–500 μg/L for all compounds. Overall-assay accuracy and precision were < 20% for the lower limit of quantification (LLOQ), except for CMP (CV=22.3%), and < 15% at other concentrations. The initial LLOQ was 20 μg/L however for CMP and DMCP it was increased to 40 μg/L. The blood volume per spot did not influence the results, but a low hematocrit (≤ 30%) was associated with a > 15% negative bias for all compounds. Punching at the perimeter of the blood spot instead of the center was associated with a positive bias. A good correlation was found between patients plasma and DBS samples of ATP, NTP and DMCP, but not for CMP. In addition, proportional differences were found. This LC-MS/MS method was analytically validated for determination of TCAs in DBS. Future validation will focus on the clinical application of the method.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Amitriptyline (ATP), nortriptyline (NTP), imipramine (IMP), and clomipramine (CMP) are first choice tricyclic antidepressants (TCAs) which are indicated by Dutch guidelines as part of therapy for anxiety or depressive disorders [1,2]. Therapeutic drug monitoring (TDM) of TCAs and their active metabolites is advised by guidelines, because these drugs display large individual differences in clearance while the therapeutic windows of these drugs are relatively small [3]. In clinical practice conventional TDM is based on venous sampling methods, however, the use of dried blood spot (DBS) sampling and analysis is gaining interest in TDM. DBS sampling originated in newborn screening to obtain an economical and rapid screening method for phenylalanine to detect phenylketonuria [4]. DBS sampling has certain advantages compared to conventional venous sampling. First, for DBS sampling blood is collected by a heel or finger puncture which is an advantage for certain vulnerable patient groups as children or elderly patients. Compared to venous sampling, DBS sampling is considered less painful and is found to be more time efficient in TDM of oral anticoagulants [5]. Furthermore, DBS samples contain low biohazard risk during transport which allows easy sampling at

* Corresponding author.
Tel.: +31 50 3638709; fax : +31 50 3632772.
** Corresponding author. Tel.: +31 522 234900.
E-mail addresses: e.j.j.berm@rug.nl (E.J.J. Berm),
maring@noorderboog.nl (J.G. Maring).

home and less expensive transport by normal postal services [6,7]. In practice, this advantage can be used in different settings to centralize TDM analysis in a safe and easy manner. Over the last decade, a rising number of DBS assays for different classes of drugs have been reported. So far, antidiabetics, immunosuppressants, analgesics, anti-HIV drugs, antihypertensive drugs, antimicrobial agents, anti-epileptics, and the iron chelator deferasirox have been included [8–25]. Among the antidepressants, a DBS assay for analysis of IMP has previously been described as well as for the SSRIs fluoxetine, reboxetine, and paroxetine, and for the atypical antidepressant venlafaxine [26–28]. However, a DBS assay for simultaneous determination of TCAs and their main active metabolites has not been reported yet. We developed an assay for simultaneous determination of ATP, NTP, IMP, and CMP as well as for the active metabolite of CMP and IMP, desmethyl-clomipramine (DCMP) and desipramine (DSP) respectively.

2. Experimental

2.1. Materials

ATP, NTP, IMP, DSP, CMP, DCMP and promazine (PMZ), all > 99%, were purchased from Sigma Aldrich (Zwijndrecht, The Netherlands). PMZ was used as internal standard. Chemical structures are shown in fig. 1. Methanol (Lichrosolv) ammonium acetate (p.a.), acetic acid 100% (p.a.) and trifluoro-acetic acid anhydride (TFAA, p.a.) were purchased from Merck (Darmstadt, Germany). Acetonitrile and purified water (ULC/MS grade) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Whatman FTK DMPK-C blood sampling cards were purchased from GE Healthcare (Hoevelaken, The Netherlands). Blank EDTA whole blood was obtained from healthy volunteers for preparation of calibration and quality control samples.

2.2. Apparatus

The LC-MS/MS consisted of an Accela autosampler and Accela pump connected with a TSQ Quantum Acces tandem mass spectrometer with an electrospray ionization source, from Thermo Scientific (Thermo, Breda, The Netherlands). System control, data acquisition, and data processing were performed using Excalibur 2.0 software from Thermo Scientific (Thermo, Breda, The Netherlands).

2.3. Chromatography

Chromatographic separation was performed on a Hypurity Aquastar column, 50 × 2.1 mm, particle size 5 μm (Thermo, Breda, The Netherlands). The separation method was similar to our previously described method for DBS analysis of venlafaxine [28]. In short, mobile phase A consisted of 2 mL TFAA, 35 ml acetic acid 100%, and 5 g ammonium acetate in 1000 ml purified water (pH 3.5). Mobile phase B was purified water, and mobile phase C, acetonitrile. The mobile phase ratio was 5% A and 95% B for two minutes. At $t=2$ minutes, the mobile phase ratio changed to 5% of A and 95% of C, in a linear slope over one minute. At $t=3$ minutes, the mobile phase ratio reverted to the starting position, and the column was allowed to equilibrate for one minute ($t=4$ min.). Including injection time, total runtime was 4.8 minutes. The flow rate was kept constant at 0.3 ml/min. The respective retention times of the TCAs and IS are given in table 1.
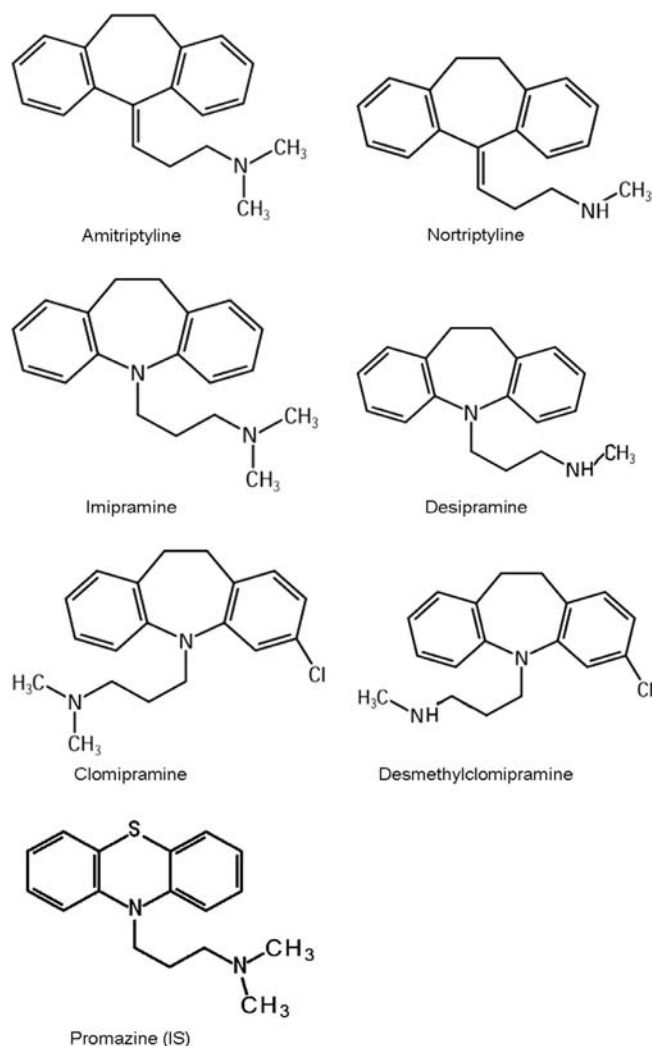
Fig. 1. Chemical structures of ATP, NTP, IMP, DPM, CPM, DCPM, and PMZ.

Table 1
LC-MS/MS conditions for TCAs.

Compound	Retention time (min)	Parent mass (m/z)	Daughter mass (m/z)	Tube Lens Voltage (V)	Collision Energy (eV)
ATP	2.48	278.2	233.3	85	17
NTP	2.42	264.2	233.3	83	15
IMP	2.43	281.2	86.3	54	16
DSP	2.35	267.2	72.4	51	15
CMP	2.55	315.2	227.2	59	38
DCMP	2.52	301.1	227.2	52	35
PMZ	2.42	285.1	86.3	56	16

2.4. Mass spectrometry

Ionization was achieved in the positive electrospray mode. Spray voltage was 3000 V. Sheath and auxiliary gas pressure were set to 40 and 10 (arbitrary units), respectively. Capillary temperature was 375 °C, and the collision gas (argon) pressure was 1.5 mTorr. The scan modus was set to selective reaction monitoring (SRM) and the mass transitions which were monitored, with their respective lens and collision voltages, are shown in Table 1.

2.5. Stock and working solutions

Stock solutions of the analytes were prepared by carefully weighting of the compounds which were individually dissolved in methanol to a concentration of 1.00 g/L. 0.5 ml aliquots from all 6 stock solutions were combined in a working solution and diluted with NaCl 0.9% to final concentrations of 10 mg/L of each analyte. The IS stock solution was prepared in methanol as well to a concentration of 0.50 g/L. To obtain the extraction solution, it was diluted with the extraction solvent to a final concentration of 40 μg/L.

2.6. Calibration and Quality control samples

Calibration samples and QC samples were prepared independently from a different stock and working solution to control for errors during preparation. Blank EDTA whole blood was spiked to obtain calibration sample concentrations of 20, 50, 100, 200, 375, and 500 μg/L and QC sample concentrations of 20 μg/L (LLOQ), 40 μg/L (QC low), 250 μg/L (QC median), and 400 μg/L (QC high) levels. With a pipette, two droplets of the spiked blood with a total volume of 50 microliters were spotted onto DMPK-C cards. The cards were left to dry overnight at room temperature. The hematocrit (Htc) of the blood which was available from the healthy volunteer was 0.44 (L/L). This blood was used without adjustments of the Htc during the further validation.

2.7. Sample preparation

During prior method optimization different extraction conditions were tested and it was found that an extraction fluid with a ratio of acetonitrile: methanol 1:3 provided the best extraction recovery of the analytes. The sample preparation method was similar to our previously described method for DBS analysis of venlafaxine [28]. In detail, circles of six millimeters were punched out by hand with a DBS puncher (Harris Uni-Core, Whatman, GE Healthcare, Hoevelaken, The Netherlands) and placed into tubes. To the tubes, 250 μL of acetonitrile:methanol 1:3 containing the IS was added, tubes were vortexed briefly and after this, shaken for five minutes. After shaking, the tubes were centrifuged at 16,000 g for 5 minutes, the supernatant was transferred to 0.5 mL auto sampler vials, the vials were closed and 5 μL was injected into the LC-MS/MS by the auto sampler.

3. Validation

Validation was performed according to the guidelines of the FDA for bio-analytical method validation [29]. The guidelines contain no specific regulations for the validations of DBS, however, it is appropriate to estimate the influence of Htc, blood spot volume, and the punch position for the validation of a DBS assay [6,30]. Therefore we analyzed influence of these variables on the analytical bias of the assay as well.

3.1. Selectivity, matrix effects, and carry over

Selectivity was validated by analyzing six blank samples from different volunteers, which were spotted on DBS cards. Chromatograms of these six blanks were compared to a spiked DBS sample, which contained the LLOQ of all compounds. The method was considered selective if other components of the matrix did not overlap with the peaks from the compounds of interest or the IS. Limits for acceptance for the noise signal from the matrix with respect to the signal of the TCA at LLOQ, was a noise signal of < 20% and with respect to the signal of the IS a noise signal of

< 5%. To investigate the matrix effect, six post-extraction calibration curves were prepared and compared to calibration curves without matrix. Blank EDTA blood from the volunteers was used to prepare 15 μL spots on pre-punched paper in a sample tube. After drying the spots were extracted with extraction solution lacking the IS. The supernatant was evaporated to dryness under a stream of nitrogen. The appropriate amounts of TCAs were added and the samples were evaporated to dryness again. Next, the extraction solution containing the IS was added and samples were analyzed. Concentrations were calculated using the analyte area/IS area ratio. All individual slopes of the calibration lines in the different matrices were compared to the slope of the calibration line obtained with the matrix free solution. The quotient of both slopes was calculated to estimate the overall bias resulting from matrix effects. Matrix effects not exceeding 10% are considered as acceptable. [12]. The relative matrix effect was calculated as the CV of the six slopes, and this value should not exceed 3-4%, although a limit of 4-5% has been previously reported [31]. Our samples were post-extraction spiked [12] which provided the disadvantage that the calculated relative matrix effects did not take into account possible differences in recovery. Carry over was analyzed by injecting two QC high samples followed by three QC low samples. The difference between the first and the third QC low sample divided by the QC high concentration was calculated as the carry over.

3.2. Linearity

Calibration samples were prepared as described under 'sample preparation'. Samples were analyzed on three consecutive days. Calibration plots were made using the peak area ratio (analyte response/IS response) versus the theoretical analyte concentration with a weighting of 1/x. Linearity of the calibration plots from each day were analyzed with linear regression and lack of fit statistics in Excel.

3.3. Within and between run accuracy and precision

Four concentrations of QC samples were prepared as described above and analyzed in five-fold in three runs ($n=15$), on consecutive days. Accuracy was estimated by calculating the bias (%) from the theoretical concentration. Variation was analyzed using one-way analysis of variance (ANOVA) to estimate -accuracy and precision.

3.4. Absolute recovery

QC samples at the low, median, and high concentration levels were prepared as described under sample preparation. Samples were compared to blank blood spots, which were post-extraction spiked, as described under matrix effect. Samples were analyzed in five-fold and the average response of the QC samples was compared with that of the post-extraction spiked samples.

3.5. Stability

Long term stability and storage conditions were asses by analyzing six QC low and high samples which were stored in the freezer (- 20 °C) up to 30 days, in the refrigerator (2 to 8 °C), and at room temperature (both up to 90 days). Samples were dried overnight and stored with a desiccant (silica) in a sealed plastic bag. QC samples were analyzed after seven days, 30 and 90 days of storage against a freshly prepared calibration line, which was dried overnight the day before the analyses. If deviations from the nominal concentration were more than 20%, stability was no longer assumed. To assess stability in the auto sampler (20 °C) a QC low and a QC high sample were injected

every 4 hours during 48 hours (13 injections in total, including $t=0\ h$). Results for stability with respect to $t=0$ were calculated (peak area ratio analyte/IS) after 12, 24, and 48 hours using linear regression analyses, If deviations were $>15\%$ stability was no longer assumed.

3.6. Effects of hematocrit

It is known that different Htc levels can influence spotted plasma volume as well as the distribution of the blood spot over the paper. Therefore influence of different Htc levels (range: 0.25 to 0.50) on the accuracy of the assay was tested [6]. For calculation of the bias caused by a varying Htc, we chose to normalize the signal (bias=0%), obtained in a blood matrix with a Htc of 0.45 (30). A Htc of 0.45 was considered close to the expected population mean [32,33]. Different Htc concentrations were prepared by centrifuging EDTA whole blood to obtain a plasma and erythrocyte fraction. The plasma fraction was spiked with the TCAs to obtain samples with TCA concentrations at QC low and QC high levels. To the spiked plasma, variable volumes of the concentrated erythrocyte fraction and blank plasma were added to obtain a range of Htc concentrations. The reported Htc values are the expected Htc values based on the calculated fractions of erythrocytes and plasma.
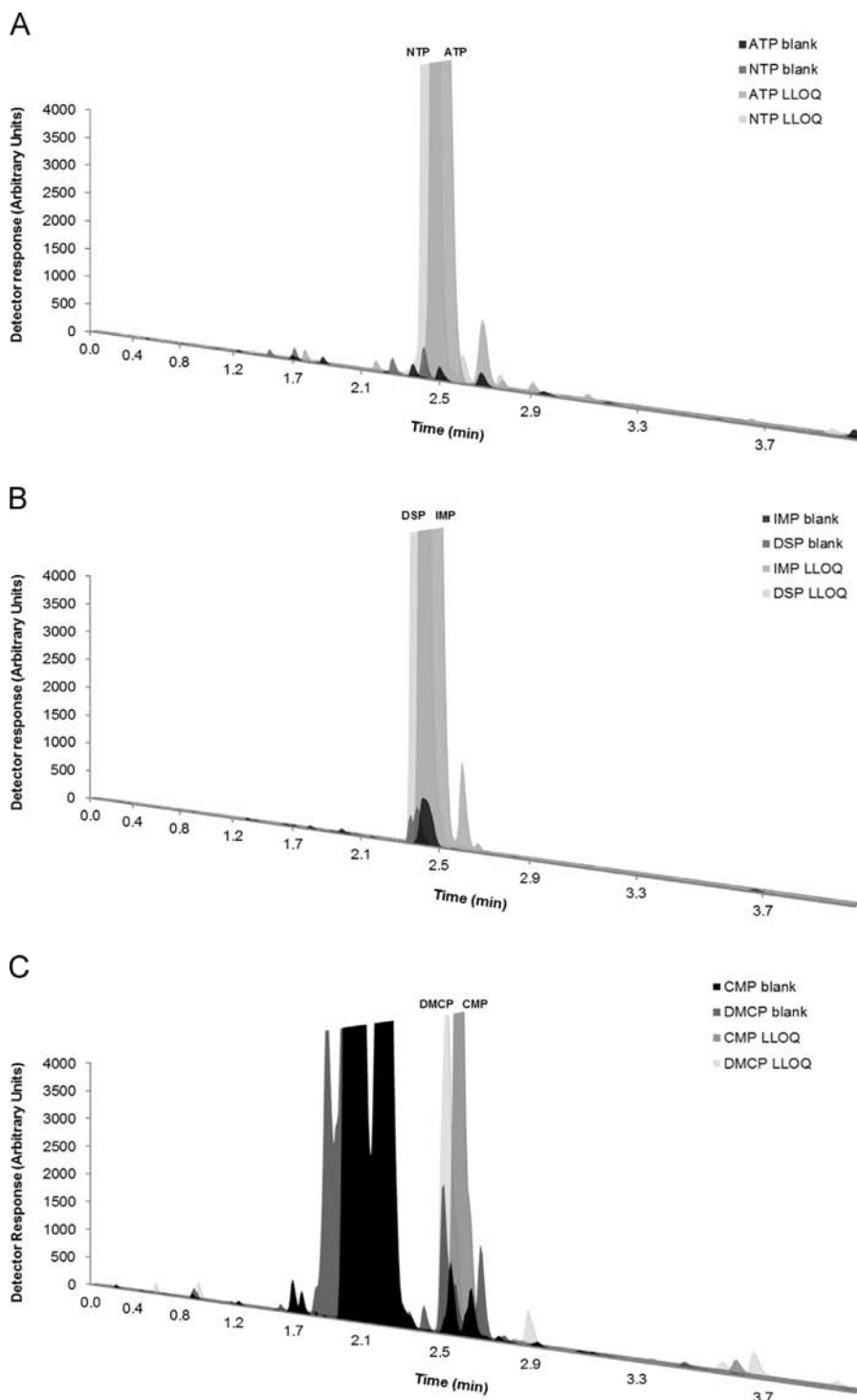

Fig. 2. Chromatograms of LLOQ and blank sample from the volunteer which gave the most interference for ATP and NTP (A), IMP and DSP (B), CMP and DCMP (C).

3.7. Effects of spotted volume and punch position

The Influence of different spotted blood volumes ranging from 20-100 μL at QC low- and QC high- level were tested. Samples were prepared in three-fold. Results were normalized to a spotting volume of 50 μL, and bias with respect to this normalized value was determined. To assess the influence of the punch position, five additional QC samples of the low and high concentrations were punched out at the center and the edge of the blood spot. Bias of the spots that were punched at the perimeter was calculated with respect to the measured concentrations of the spots punched at the center.

3.8. Clinical validation

Patients visiting the laboratory for routine blood analysis of a TCA for TDM purposes, were invited to participate in the clinical validation of the DBS method. If patients gave informed consent, an extra blood sample was collected by finger prick on a DBS card. The plasma samples were analyzed by a fully validated routine LC-MS/MS method, which has proven to be robust and reliable in external quality control programs. The correlation between plasma and DBS samples was assessed with linear regression analysis in Excel. Patient samples sometimes originated from the same patient, however, if this was the case samples were collected on different days. Approval for this study was obtained from the Medical Ethics Committee of Diaconessen Hospital Meppel.

4. Results

4.1. Matrix effects, selectivity and carry over

Visual inspection of the chromatograms indicated there was interference from the blank blood spots for DMCP (fig. 2). For the other compounds, no interference was detected. The highest co-eluting peak was observed for DCMP, which was 26.1% of the peak area of the LLOQ. For the other compounds co-eluting peak area was < 20%. For the IS the highest co-eluting peak was 0.2%.
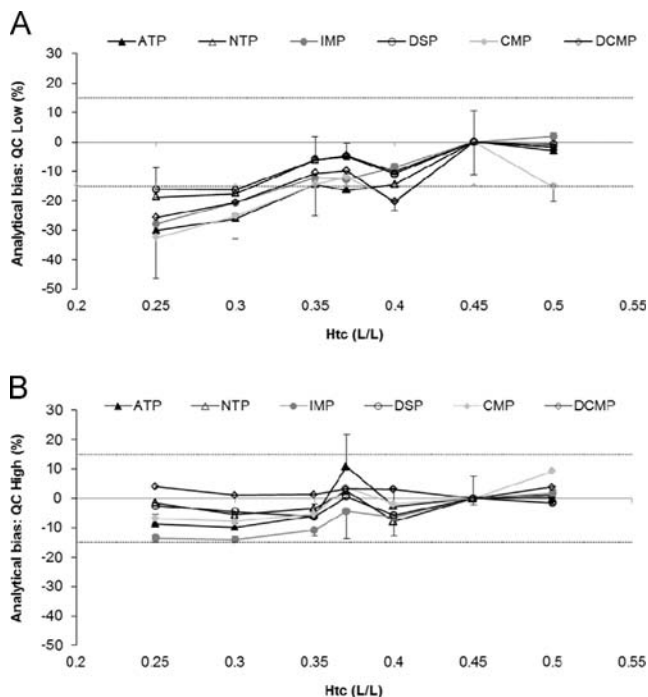
Matrix effects are summarized in table 2. For the active metabolites (NTP, DSP, and DCMP) a moderate negative matrix effect (ranging from -14.2 to -11.3%) and a relative matrix effect

Table 2
Validation results of matrix effects, specificity, linearity, recovery and stability.

Validated parameter	Results for respective compound					
	ATP	NTP	IMP	DSP	CMP	DCMP
Matrix effects [n=6]						
Average bias (%) of slopes with respect to neat solution	-3.8	-14.2	-6.7	-13.5	-4.3	-11.3
Range % bias	-10.9 to 2.3	-20.4 to -3.6	-12.7 to -2.7	-19.3 to -1.0	-11.2 to -0.5	-16.3 to -3.1
CV (%) of slopes (relative matrix effect)	4.6	7.0	4.2	7.5	4.1	5.3
Overall linearity [n=18] - r^2	0.990	0.994	0.994	0.996	0.990	0.990
Recovery (%)(RSD %) [n=5]						
Low	81 (14)	101(10)	83(10)	101(9)	83(8)	98 (20)
Medium	104 (14)	123(13)	98 (12)	115(13)	101 (12)	110. (19)
High	97(5)	113 (6)	94 (5)	108(5)	95 (8)	112 (11)
Auto sampler stability (recovery %) [n=13]						
12 h	107.0	106.1	106.7	103.1	109.8	106.8
24h	114.0	112.1	113.4	106.2	119.6	113.7
48 h	128.1	124.1	126.8	112.4	139.1	127.3

Table 3
Validation results of within, between as well as overall run accuracy and precision. $n=15$, except for CMP ($n=14$ due to IS error).

TCA	ATP	NTP	IMP	DSP	CMP	DCMP	Limits
Repeatability (RSD %)							
LLOQ	10.4	8.1	6.7	12.3	20.1	19.7	
Low	10.3	6.5	5.9	9.6	14.1	12.7	
Medium	6.6	5.1	6.4	5.2	7.7	8.0	
High	4.4	6.2	4.0	4.3	4.8	7.6	
Intermediate precision (RSD %)							LLOQ < 20% L, M, H < 15%
LLOQ	10.4	8.1	6.7	18.6	22.3	19.7	
Low	10.3	6.5	5.9	10.2	14.1	13.4	
Medium	8.2	5.1	8.3	5.5	7.9	8.0	
High	6.9	6.2	6.6	4.3	5.9	7.7	
Accuracy within-run run 1, 2, and 3 (% bias)							
LLOQ	-3.2;-3.5; -7.5	-1.2; -2.2; -2.5	2.9; -1.1; 3.4	-8.2; -1.6; 21.5	-12.7; 6.1; 13.7	-10.0; -14.6; -21.7	
Low	-7.8; -6.1; -7.2	-2.7; -1.1; -0.1	-4.2; 0.6; -0.7	-6.0; -3.5; 4.5	4.7; 4.1; 15.3	3.3; -5.6; -10.3	
Medium	-2.1; 9.0; 0.1	3.3; 6.0; 5.8	-0.9; 11.1; 2.0	-0.7; 5.1; 1.3	2.9; 7.6; 11.3	-1.4; 1.2; -2.6	
High	-0.5; 8.0; -3.0	6.4; 3.9; 2.3	-0.6; 10.3; 1.7	0.3; 2.8; -0.7	2.0; 7.1; 10.6	3.6; 3.3; -3.2	
Accuracy between-run/overall (% bias)							LLOQ < 20% L, M, H < 15%
LLOQ	-4.7	-2.0	1.7	3.9	5.2	-15.4	
Low	-7.1	-1.3	-1.4	-1.7	8.0	- 4.2	
Medium	2.3	5.0	4.1	1.9	7.3	-0.9	
High	1.5	4.2	3.8	0.8	6.6	1.2	

Fig. 3. Effects of Htc on analytical bias (%) at QC low (A) and QC high level (B). For visual purposes, error bars are only shown for (A) DSP and CMP, (B) ATP and IMP. $n=3$ for each point, except for QC low Htc=0.37 [n= 2], due to IS error.

ranging from 5.3 to 7.5% was observed. For the parent compounds IMP and CMP, no matrix effects were detected, however for ATP a relative matrix effect of 4.6% was found. No carry over was observed (range:-1.32 to -0.24%).

4.2. Linearity, recovery, stability, accuracy and precision

Results of linearity, recovery and stability tests are summarized in table 2. The assay was found to be linear from 20-500 µg/L ($r^2 > 0.990$), and no significant lack of fit was found for any of the TCAs. The recovery was high (> 80%) for all compounds.

At the different storage conditions, we observed a positive bias (range: 23- 33%) for all compounds after seven days and one month of storage in the freezer. When stored at room temperature, all compounds were stable for three months, except for the QC high samples of DCMP (bias: -23.8%) which was stable up to one month. After three months of storage in the refrigerator all samples were found stable as well. All TCAs were stable in the auto sampler up to 12 hours. Up to 24 hours, all compounds were considered stable except for CMP.

None of the TCA's displayed acceptable stability for longer periods as up to 48 hours in the autosampler.

Within-, between- and overall-run accuracy and precision are summarized in table 3. Precision was within the limits of acceptance, except for the within- and overall-run precision of the LLOQ of CMP. Overall accuracy was within the limits of acceptance for all compounds.

4.3. Effects of hematocrit, spotted blood volume and punch position

Overall, the Htc effects were more pronounced in the QC low samples compared to the QC high samples. Effects of Htc on analytical bias are shown in fig. 3. In the QC low samples, a low hematocrit (≤ 0.30 L/L) was associated with a > 15% negative bias for all compounds. Parent compounds (ATP, IMP, CMP) were more sensitive for bias at a low Htc compared to their metabolites (NTP, DSP, DCMP). At higher Htc levels (0.35- 0.40 L/L) a bias > +/- 15%
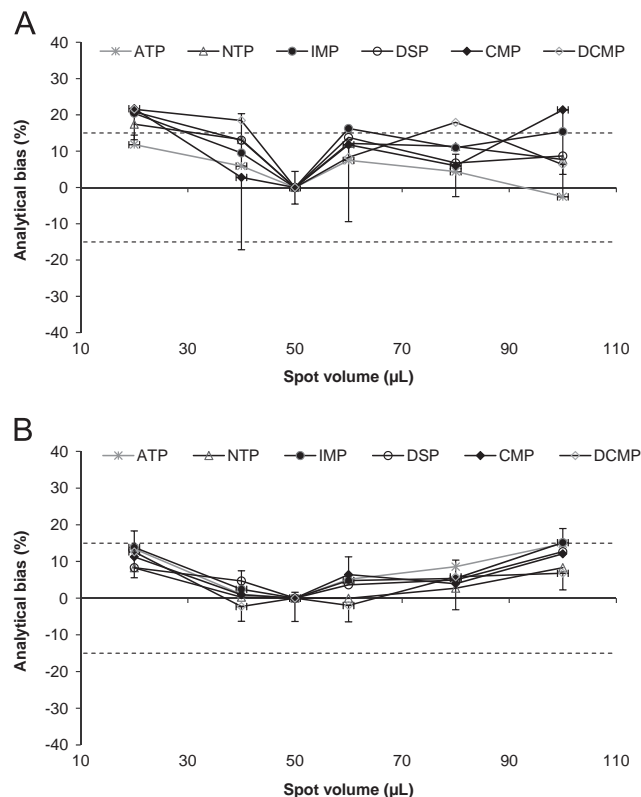
Fig. 4. Effect of spotting volume on analytical bias (%) at (A) QC Low and (B) QC high. For visual purposes, error bars are only shown for (A) CMP and DCMP (B) CMP and IMP. $n=3$ for each point.
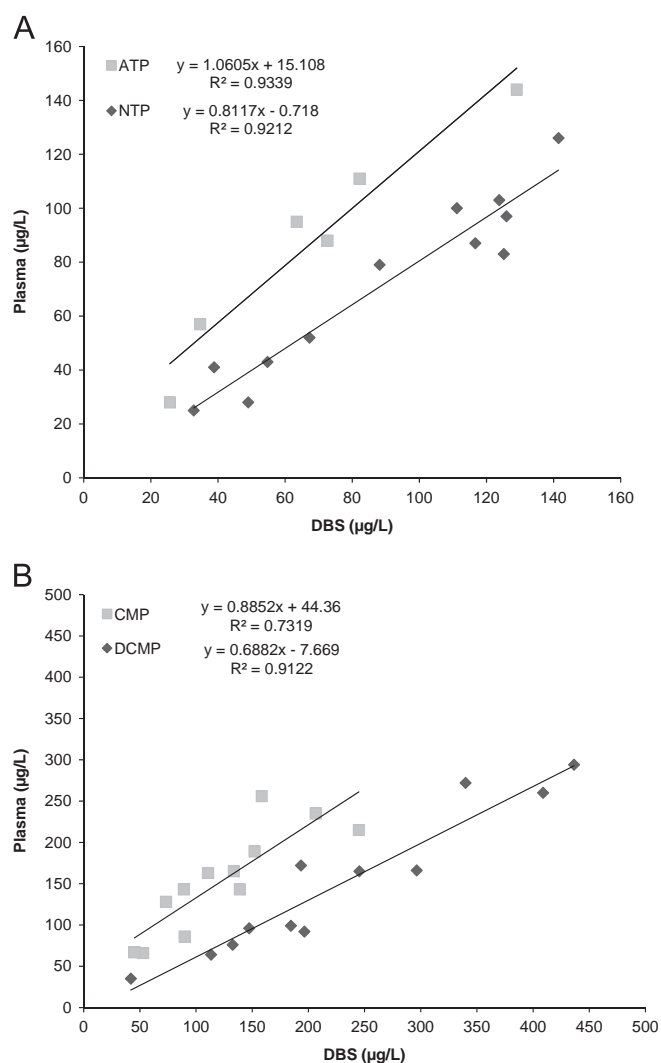
was observed for ATP, CMP, and DMCP. The effects of the spotting volume on analytical bias for the QC low samples are shown in fig. 4. Different spot volumes induced bias for all compounds and a higher bias was found if spot volume was small (20 µL) or high (100µL). There was no trend observed in the effect of the spot volume on the analytical bias. Punch position influenced the result. If the punch position was in the middle of the blood spot, the measured concentration was more accurate. Bias introduced in the QC low samples by punching at the edge of the spot ranged from 9.5 to 23.5%. For the QC high samples, analytical bias was smaller and ranged from 6.1 to 10.9%.

4.4. Clinical validation

Due to limited routine TDM of ATP, only seven patient samples were available for analysis. For one of these samples a concentration below the LLOQ was found in both plasma as well as in DBS, leaving six remaining samples from four different patients for further analysis (fig. 5A). For IMP, no samples were available due to lack of routine TDM samples of IMP. The metabolite, DSP is not on the Dutch market as a separate drug, therefore no samples were available for DSP as well. For the other compounds, 12 samples were analyzed from 11 different patients for NTP (fig. 5A) and nine different patients for CMP and DCMP (fig. 5B). A good correlation was found for ATP ($r^2=0.93$), NTP ($r^2=0.92$), and DCMP ($r^2 =0.91$), however for CMP the correlation was much lower ($r^2=0.73$).

5. Discussion & Conclusion

An assay to determine concentrations of TCAs in DBS samples was developed and tested against the requirements in the FDA

Fig. 5. Correlation between patients plasma and DBS samples for ATP and NTP (A), CMP and DCMP (B). $N=12$, except for ATP where $N=6$.

stored up to three months in the refrigerator, and up to one month at room temperature. Auto sampler stability of extracted samples was limited till 12 h, due to increasing response ratio's over time. This increase was caused by a decreasing signal from the IS combined with a slightly increasing signal of the analytes. Unfortunately, we were unable to find an adequate explanation for this phenomenon.

The bias caused by variation in Htc, blood spot volume and punch position was quantified. Although so far no limits for acceptance have been defined for these variables, it is reasonable to correct for Htc effects if bias exceeds 15% [35]. At lower Htc levels, a negative bias was found for all compounds at QC low concentrations. This is assumed to be related to the lower viscosity of the blood at a lower Htc [30]. Based on the expected Htc in the average psychiatric patient population a $> +/-$ 15% bias at a Htc of > 0.35 was considered unacceptable. Results suggested such biases could occur at low concentrations of ATP, CMP, and DMCP. Therefore corrections for Htc should be further investigated. Blood spot volume did also affect analytical bias, however there was no general trend in these effects for all TCAs. The observed biases are probably related to normal analytical variation. Effects of punch position on analytical bias was limited, however it was found to be more accurate to punch in the middle of the blood spot for all TCAs. If possible, we recommend punching in the middle of the blood spot. Patient samples indicated a good correlation between plasma and DBS samples for ATP, NTP and DCMP. For CMP the correlation was less pronounced and a correction for Htc should be studied during further validation by measuring Htc in DBS as was recently demonstrated by Capiau et al. [36]. Moreover, results suggested a proportional difference in concentrations between plasma and DBS samples. To be able to translate DBS result to plasma values, a correction factor seems therefore indicated. In addition, the assumption of a linear relationship should be tested.

This is the first assay, which describes a DBS method for TDM of common TCAs. Only for IMP, Déglon et al. have previously described a DBS assay, but their report did not include a full validation of the assay [27]. We investigated effects of Htc, spotting volume, and punch position, which were not reported before. Several assays for the simultaneous determination of TCAs, which make use of blood plasma obtained from venous puncturing are reported [37–39]. Compared to these methods, our DBS assay is performing similar in terms of accuracy, and precision, however our LLOQ is somewhat higher. The total chromatographic run time of our assay is comparable as well. We consider it advantageous that our method does not involve any pretreatment step like solid-phase extraction and is restricted to a few simple extraction steps. Nevertheless, additional bias from varying Htc, blood spot volume and punch position, would make venous puncturing more accurate for determination of TCAs in high precision pharmacokinetic research.

For TDM, this DBS assay is analytically validated and it appears a promising alternative sampling method in TCA treated patients. Compared to current methods, reliability of the determination of ATP, CMP, and DCMP might be decreased at lower concentrations, due to Htc effects. However, this assay still provides clinical valuable information, and it has many advantages for the patient as well as for sample logistics, as stated before. Based on the clinical needs at a certain setting, different sampling approaches might be considered. Further clinical validation of this assay is still ongoing to determine possible correction factors for conversion of DBS to plasma values as well as Htc corrections. This assay is designed to be used for the determination of NTP in a Dutch multicenter trial (CYSCE Trial: ClinicalTrail.gov Identifier NCT01778907), in which the effect of CYP2D6 genotyping combined with TDM on time to reach adequate blood drug levels is investigated, in older patients. The possibility of

guideline. For ATP, NTP, IMP, and DSP requirements were met, however for CMP and DMCP, the LLOQ of 20 µg/L did not comply with the requirements concerning selectivity and precision. As a result of this validation, we therefore decided to adjust the LLOQ for these compounds to 40 µg/L. It should be notified, that the higher LLOQ of 40 µg/L for DCMP has not been actually tested with respect to the requirements for selectivity, however based on our findings we assume that since the concentration is doubled from 20 to 40 µg/L, the found signal to noise ratio of 26.1% will reduce to a ratio of $< 20\%$. In addition, by using the QC low (40 µg/L) samples of CMP and DMCP as the new LLOQ samples for determination of the precision, the experiment for the precision did no longer contain the 4 concentration levels subscribed by the guidelines. However, based on the results found at 20 µg/L, which were only slightly deviating for CMP ($CV=22.3\%$) and within limits for DCMP ($CV= 19.7\%$), we consider this acceptable.

Moderate matrix effects were observed for the active metabolites which could be related to their chemical structure. All active metabolites have a free nitrogen atom which is not present in the parent compounds and IS. This makes the active metabolites more polar and therefore probably more sensitive to ion suppression with respect to the IS [34]. Although we observed matrix effects during validation, these effects were considered acceptable, within the scope of TDM purposes. Dried blood spots could at least be

taking a blood sample by a trained nurse or physician with a DBS, is of great advantage, because it offers more flexibility, which is often a problem in clinical trials.

Acknowledgements

References

[1] MJ van Avendonk, LJ Hassink-Franke, B Terluin, HW van Marwijk, T Wiersma, JS. Burgers, Ned Tijdschr Geneeskd. 156 (34) (2012) A4509.
[2] M van Avendonk, E van Weel-Baumgarten, G van der Weele, T Wiersma, JS. Burgers, Ned Tijdschr Geneeskd. 156 (38) (2012) A5101.
[3] N. Van Brunt, Ther Drug Monit. 5 (1) (1983) 1–10.
[4] R GUTHRIE, A. SUSI, Pediatrics 32 (1963 Sep) 338–343.
[5] K Woods, JD Douketis, T Schnurr, K Kinnon, P Powers, MA. Crowther, Thromb Res. 114 (3) (2004) 161–165.
[6] PM Edelbroek, J van der Heijden, LM. Stolk, Ther Drug Monit. 31 (3) (2009 Jun) 327–336.
[7] N Spooner, Y Ramakrishnan, M Barfield, O Dewit, S. Miller, Bioanalysis 2 (8) (2010) 1515–1522 (Aug).
[8] S Aburuz, J Millership, J. McElnay, J Chromatogr B Analyt Technol Biomed Life Sci 832 (2) (2006) 202–277 (Mar 7s).
[9] J van der Heijden, Y de Beer, K Hoogtanders, M Christiaans, GJ de Jong, C Neef, et al., J Pharm Biomed Anal. 50 (4) (2009) 664–670 (Nov 1).
[10] AJ Wilhelm, JC den Burger, A Chahbouni, RM Vos, A. Sinjewel, J Chromatogr B Analyt Technol Biomed Life Sci 877 (30) (2009 Nov 15) 3916–3919.
[11] AJ Wilhelm, JC den Burger, RM Vos, A Chahbouni, A. Sinjewel, J Chromatogr B Analyt Technol Biomed Life Sci 877 (14-15) (2009 May 15) 1595–1598.
[12] JC den Burger, AJ Wilhelm, A Chahbouni, RM Vos, A Sinjewel, EL. Swart, Anal Bioanal Chem. 404 (6-7) (2012) 1803–1811 (Oct).
[13] RN Rao, PK Maurya, M Ramesh, R Srinivas, SB. Agwane, Biomed Chromatogr. 24 (12) (2010 Dec) 1356–1364.
[14] EJ Oliveira, DG Watson, NS. Morton, J Pharm Biomed Anal. 29 (5) (2002) 803–809 (Jul 31).
[15] CF Clavijo, KL Hoffman, JJ Thomas, B Carvalho, LF Chu, DR Drover, et al., Anal Bioanal Chem. 400 (3) (2011 May) 715–728.
[16] W Kromdijk, JW Mulder, H Rosing, PM Smit, JH Beijnen, AD. Huitema, J Antimicrob Chemother. 67 (5) (2012 May) 1211–1216.
[17] JT Hoffman, SS Rossi, R Espina-Quinto, S Letendre, EV. Capparelli, Ther Drug Monit. 35 (2) (2013 Apr) 203–208.
[18] RN Rao, SS Raju, RM Vali, GG. Sankar, J Chromatogr B Analyt Technol Biomed Life Sci (2012 Aug) 47–541;902 (2012 Aug) 47–54.
[19] ML Della Bona, S Malvagia, F Villanelli, E Giocaliere, D Ombrone, S Funghini, et al., J Pharm Biomed Anal. 5 (78-79) (2013 May) 34–38.
[20] TM Reddy, CI Tama, RN. Hayes, J Chromatogr B Analyt Technol Biomed Life Sci 879 (30) (2011 Nov 15) 3626–3638.
[21] DH Vu, RA Koster, JW Alffenaar, JR Brouwers, DR. Uges, J Chromatogr B Analyt Technol Biomed Life Sci 879 (15-16) (2011) 1063–1070 (May 1).
[22] DH Vu, MS Bolhuis, RA Koster, B Greijdanus, WC de Lange, R van Altena, et al., Antimicrob Agents Chemother. 56 (11) (2012 Nov) 5758–5763.
[23] T Vnucec Popov, LC Maricic, H Prosen, DB. Voncina, Biomed Chromatogr. (2013 Mar) 26.
[24] NM Shah, AF Hawwa, JS Millership, PS Collier, JC. McElnay, J Chromatogr B Analyt Technol Biomed Life Sci 1 (923-924) (2013) 65–73 (Apr).
[25] R Nirogi, DR Ajjala, V Kandikere, R Aleti, S Srikakolapu, H. Vurimindi, J Chromatogr B Analyt Technol Biomed Life Sci (2012) 65–73 (Oct 15)907 (2012) 65–73.
[26] J Deglon, E Lauer, A Thomas, P Mangin, C. Staub, Anal Bioanal Chem. 396 (7) (2010) 2523–2532 (Apr).
[27] J Deglon, A Thomas, A Cataldo, P Mangin, C. Staub, J Pharm Biomed Anal. 49 (4) (2009) 1034–1039 (May 1).
[28] EJ Berm, E Brummel-Mulder, J Paardekooper, E Hak, B Wilffert, JG. Maring, Anal Bioanal Chem. 406 (9-10) (2014 Apr) 2349–2353.
[29] US food and drug administration, bioanalytical method validation [Internet].; 2001. Available from: ⟨http://www.fda.gov/downloads/Drugs/Guidances/ucm070107.pdf⟩.
[30] W Li, FL. Tse, Biomed Chromatogr. 24 (1) (2010) 49–65 (Jan;).
[31] BK. Matuszewski, J Chromatogr B Analyt Technol Biomed Life Sci 830 (2) (2006) 293–300.
[32] M Maes, Vyvere J Van de, E Vandoolaeghe, T Bril, P Demedts, A Wauters, et al., J Affect Disord. 40 (1-2) (1996) 23–33 (Sep 9).
[33] CH Alves de Rezende, LM Coelho, LM Oliveira, N. Penha Silva, J Nutr Health Aging. 13 (7) (2009 Aug) 617–621.
[34] R Bonfiglio, RC King, TV Olah, K. Merkle, Rapid Commun Mass Spectrom. 13 (12) (1999 Jun) 1175–1185.
[35] P Timmerman, S White, Z Cobb, R de Vries, E Thomas, B van Baar, Bioanalysis (2013 Jul) 5.
[36] S Capiau, VV Stove, WE Lambert, CP. Stove, Anal Chem. 85 (1) (2013) 404–410 (Jan 2).
[37] AJ Santos-Neto, J Bergquist, FM Lancas, PJ. Sjoberg, J Chromatogr A. 1189 (1-2) (2008) 514–522 (May 2).
[38] J. Hasselstrom, J Chromatogr B Analyt Technol Biomed Life Sci 879 (1) (2011) 123–128.
[39] A de Castro, M Ramirez Fernandez Mdel, M Laloup, N Samyn, G De Boeck, M Wood, et al., J Chromatogr A. 1160 (1-2) (2007 Aug 10) 3–12.